

Features Of Apoptosis Regulation In Hepatic Pathology And The Role Of Inducers Of The Monooxygenase And Nitric Systems Of Hepatocytes

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Abstract

In a study on male white outbred rats with a body weight of 180-250 g, it was found that animals with OTG showed higher levels of P53 and TNF α , as well as higher levels of cytochrome C release from mitochondria. The administration of benzonal, an inducer of drug metabolism, at a dose of 50 mg/kg to animals with OTG (CCl₄) significantly reduced the symptoms of apoptosis. On the other hand, the administration of cimetidine at a dose of 10 mg/kg stimulated the apoptosis of hepatocytes, which dramatically increased the levels of P53, TNF α , and cytochrome C in biological fluids. In animals with OTG, arginine, an NO L-inducer, reduced the pro-apoptotic indicators. Compared to the OTG group, the non-selective NOS inhibitor L-NAME increased the levels of P53 protein, TNF α , and cytochrome C release from hepatocyte mitochondria.

Keywords: Pathophysiology, liver pathologies, NO – system with monooxygenase, cytochrome P-450, Endothelial (eNOS), neuronal (nNOS), inducible (iNOS).

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1. Introduction

The liver belongs to the stress-sensitive organs capable of regeneration after damage, due to cellular cooperation, the presence of molecular mechanisms of the acute phase reaction and the synthesis of a number of molecules of a protective nature. Apoptosis of hepatocytes is a key mechanism of cell death involved in the physiological renewal and pathology of the liver. In hepatitis, dysregulation of apoptosis contributes to increased inflammation, fibrosis progression, and poor liver function. A violation of the balance between signaling pathways regulating programmed cell death has been described in studies on the pathogenesis of hepatitis of various etiologies [1,2].

The nitric system, in particular nitric oxide (NO) production, is an important regulator of vascular function, inflammation, and cellular response to injury. Recent studies have shown that impaired activity of the nitric system affects the development of inflammation and apoptosis in the liver tissue, acting as a factor of adaptation or damage, depending on the level and conditions of NO formation. There is evidence that the pro-apoptotic effect of NO on liver tissue is realized directly through the mechanisms of caspase activation [3,5]. One of the ways of activation of caspases consists in the release of cytochrome C and other factors from the mitochondria into the cytoplasm [4]. This release is prevented by the anti-apoptotic Bcl-2 and Bcl-LXL

proteins and activated by the pro-apoptotic Bax protein [8]. In the cytoplasm, cytochrome C binds to two proteins, Apaf-1 and procaspase-9, leading to activation of caspase-9 and caspase-3, causing apoptosis [1,8]. One of the mechanisms of cytochrome C release from mitochondria is associated with the opening of MPT pores (mitochondrial permeability transmission pores) [9, 11]. It has been shown that activation of MPT pores occurs under the action of ONOO- and, therefore, this may be one of the mechanisms by which NO activates apoptosis [11]. Another mechanism of NO influence on cytochrome C release is due to a change in the ratio between Bcl-2, Bcl-XL and Bax. Finally, NO can activate apoptosis through transcription factors such as AP-1, NFkB, CREB, p53, and early genes [6,7,10].

Thus, a comprehensive study of apoptosis in hepatitis, taking into account the effect of MOS inducers and inhibitors, as well as the role of the nitric system, is an urgent scientific problem. The results obtained can contribute to the deepening of knowledge of the molecular mechanisms of liver damage and the development of new pathogenetically based approaches to the prevention and treatment of hepatitis.

Based on the literature data, the objective of our study was to study the level of pro-apoptotic parameters under the action of an inducer and inhibitor of the monooxygenase system in hepatocyte microsomes in animals with AH caused by CCl₄.

2. Methods

Studies were conducted on 48 white mongrel male rats with a body weight of 180-250 g. Toxic liver damage was reproduced by subcutaneous four-fold injection of CCl₄ (50% oil solution) at a dose of 0.4 ml/100 g of animal body weight according to the recommendations of N. Kh. Abdullayev and Kh. Ya. Karimov (1986). Depending on the administered drugs, the animals were divided into 5 groups: Group 1 received benzonal at an effective dose of 75 mg / kg, group 2 received cimetidine at a dose of 10 mg / kg, group 3 received L-NAME at a dose of 10 mg / kg, group 4 received L-arginine 50 mg / kg, Group 5 consisted of animals with CCl₄ treated with water. The drugs were injected intragastrically for 6 days. Groups of intact animals were used as controls.

The liver was perfused through the inferior vena cava with a cooled (0±4 ° C) 50 mM Tris HCL buffer, pH 7.4, containing 0.05 M KCl and 0.25 M sucrose. After washing the liver from blood, it was crushed and

homogenized in the same solution (1: 3). Microsomes were precipitated from the postmitochondrial fraction obtained by centrifugation on VAC-602 (Germany) after 20 min of unscrewing at 12 thousand g at 105 thousand g. g for 60 minutes. All procedures were performed in a KHS-12 refrigerator (Russia) at 0±4 ° C. In microsomes resuspended in 100 mM Tris-HCl buffer, pH 7.4, the activity of the monooxygenase system was evaluated by the content of cytochrome P-450, using the classical method of T. Omura, and R. Sato (1964).

Nitrooxygenase activity was determined by the content of stable metabolites of nitrites and nitrates NO-NO₂- and NO₃- - according to the method of P. P. Golikov et al. (2000), endothelial NOS (eNOS) activity according to V. V. Sumbaeva, I. M. Yasinskaya (2000), inducible NOS (iNOS) and peroxynitrite (ONO₂-) concentrations according to M. Yu. Ravaeva, Ye. N. Chuyan (2011), the content and activity of monooxygenase and oxidoreductases of the nitrooxygenase system were recorded on a computerized two-beam laser. UV-2100 spectrophotometer (Ltd, China). The content and activity of oxidoreductases were calculated in microsomes per milligram of protein in 1 ml (mg / ml), which was determined by the method of O. N. Lowry et al. (1951). Enzyme-linked immunosorbent assay was used to determine serum levels of P53, TNF-α, and cytochrome C according to Gvatua et al. (1990).

The obtained results were subjected to statistical processing using the Excel application software package Excel, Statistical for Windows V. 6,0. The distribution of samples was carried out on the basis of the Student's criterion (t) with the calculation of the error probability (P). The relationship between the indicators was determined using Pearson correlation analysis (r). The data were considered reliable at p<0.05.

3. Results And Discussion

The conducted studies showed an increase in the content of P53 protein in rats with carbon tetrachloride hepatitis by 1.35 (P<0.05) times relative to the indicators of intact rats (Table 5.11). At the same time, we observed an increased release of cytochrome c into the blood serum, which was accompanied by an increase in its level in the blood serum by 1.32 (P<0.05). At the same time, there was also a 1.29-fold (P<0.05) increase in the content of TNF-α in the blood serum relative to intact rats. Such changes in apoptosis parameters occurred against the background of a significant decrease in the content of cytochrome P450, suppression of endothelial nitric oxide

synthase, and induction of its inducible form. This led to an increase in the content of nitric oxide and peroxynitrite by 1.8 ($P<0.01$) and 2.25 ($P<0.001$) times, respectively, relative to the values of intact rats.

Therefore, it can be said that acute liver damage with carbon tetrachloride activates the processes of apoptosis of hepatocytes, which coincides with high values of inducible nitric oxide synthase and products of its free radical oxidation.

Injection of benzonal to rats with acute carbon tetrachloride hepatitis in optimal doses significantly reduced the content of protein P53, cytochrome c, and TNF- α by 1.24 ($P<0.05$), 1.26 ($P<0.05$), and 1.17 ($P<0.05$) times, respectively, relative to the values of rats with AH and approaching the values of intact animals. At the same time, an increase in the content of cytochrome P450 and a decrease in the activity of iNOS and its metabolic products were noted, although their complete recovery was not observed.

Injection of the MOC-cimetidine inhibitor further induced apoptosis. Thus, the content of protein P53,

cytochrome c, and TNF- α statistically significantly increased by 1.21 ($P<0.05$), 1.33 ($P<0.05$), and 1.24 ($P<0.05$) times, respectively, relative to the values of rats with AH. Activation of the mitochondrial apoptosis pathway was found. The above parameters significantly exceeded the values of intact rats by 1.63 ($P<0.01$), 1.75 ($P<0.01$) and 1.6 ($P<0.01$) times, respectively. Along with this, an even greater inhibition of cytochrome P450, e-NOS activity, and a sharp increase in the activity of iNOS and its metabolic products were noted.

MOS inhibitors are widely used in clinical practice in the treatment of many liver diseases [10, 11]. At the same time, it remains unclear due to what mechanisms their toxic effect develops [11]. A number of researchers associate the development of the toxic effect of MOS inhibitors through the mechanisms of induction of reactive oxygen species and, above all, the effect on the activity of the nitric system [5]. The MOS inhibitor cimetidine activates the processes of the nitrate reductase system, increasing the formation of toxic oxygen metabolites and, as a consequence, apoptosis processes.

Table 5.12

Activity of monooxygenases, the nitric system, and pro-apoptotic drugs. parameters in microsomes of hepatocytes in animals with CCl₄-induced liver damage under the action of inducers and inhibitors, $M\pm m$

Groups	P-450 groups, nm / mg	NO, $\mu\text{m}/\text{mg}$	eNOS, $\mu\text{m}/\text{min}/\text{mg}$	iNOS, $\mu\text{m}/\text{min}/\text{mg}$
Intact	0,97 \pm 0,031	5,5 \pm 0,16	17,4 \pm 0,63	0,10 \pm 0,002
AH	0,53 \pm 0,012***	10,0 \pm 0,37***	8,55 \pm 0,29***	0,21 \pm 0,009 ***
AH+B	1,10 \pm 0,043*^^^	7,8 \pm 0,23***^^^	9,6 \pm 0,38***^	0,18 \pm 0,006***^
AH+C	0,31 \pm 0,01***^^^	11,8 \pm 0,45***^^^	5,8 \pm 0,19***^^^	0,24 \pm 0,010***
AH+L-Arginine	0,71 \pm 0,015***^^^	8,3 \pm 0,29***^	9,6 \pm 0,35***^	0,16 \pm 0,004***^
AH+L-NAME	0,41 \pm 0,011***^^^	13,3 \pm 0,57***^^^	6,2 \pm 0,18***^^^	0,26 \pm 0,010***^

Groups	ONO2-groups, $\mu\text{m}/\text{mg}$	P53, pg/ml	Cit. C, nmol/l	TNF α , pg/ml
Intact	0,08 \pm 0,002	0,68 \pm 0,016	0,81 \pm 0,046	15,3 \pm 0,84
AH	0,18 \pm 0,007***	0,92 \pm 0,029***	1,07 \pm 0,061**	19,7 \pm 0,97**
AH +B	0,15 \pm 0,006***^^	0,74 \pm 0,018*^^^	0,85 \pm 0,043^	16,8 \pm 0,88^
AH +C	0,22 \pm 0,009***^^	1,11 \pm 0,037***^^	1,42 \pm 0,072***^^	24,5 \pm 1,06***^^
AH+L-arginine	0.13 \pm 0.004** * ^^	0,72 \pm 0,021^^^	0,87 \pm 0,068	16,5 \pm 0,79^
AH +L-NAME	0,23 \pm 0,007***^^	1,08 \pm 0,047***^	1,32 \pm 0,075***^	25,7 \pm 1,05***^^

Note: * - differences with respect to the data of the intact group are significant (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$); ^ - differences with respect to the data of the intact group are significant (^ - $P < 0.05$, ^^ - $P < 0.01$, ^^ ^ - $P < 0.001$)

The NO system is aimed at maintaining NO homeostasis in tissues, organs, and systems with the participation of a family of cytochrome P-450 – like enzymes that use the amino acid L-arginine as a substrate [9, 10]. All isoforms of NO synthases – endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) – use L-arginine, oxygen, and NADPH as substrates for NO synthesis [11]. Cofactors of the catalytic activity of NOS isoforms are tetrahydrobiopterin (HH44), calmodulin, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMH) [1]. It was noted that a decrease in the level of L-arginine and HH44 initiates iNOS, nNOS, and eNOS to produce the superoxide anion radical and the formation of reactive oxygen species [11]. An important role in this process is played by overexpression of NO [14], which is associated with inactivation of DNA damage enzymes and acceleration of apoptosis processes. At the same time, MOS, which is mainly responsible for cell homeostasis in combination with antioxidants for the detoxification of ROS and superoxide, also uses oxygen and NADPH in its reactions, i.e. the same cofactors as NOS. Despite the importance of these systems in maintaining the physicochemical homeostasis of cells, their functional relationship at the level of microsomal liver oxidation remains completely unclear.

Injection of L-arginine to rats with AH in optimal doses significantly reduced the content of protein P53, cytochrome c, and TNF- α in the blood serum of experimental animals by 1.28 ($P < 0.05$), 1.23 ($P < 0.05$),

and 1.18 ($P < 0.05$) times, respectively, relative to the values of rats with AH and the approximation of indicators of intact animals. At the same time, an increase in the content of cytochrome P450 and a decrease in the activity of iNOS and its metabolic products were noted, although their complete recovery was not observed, since the studied parameters significantly differed from the values of intact rats.

The injection of L-NAME further induced apoptosis processes. Thus, the content of protein P53, cytochrome c, and TNF- α statistically significantly increased by 1.5 ($P < 0.05$), 1.52 ($P < 0.05$), and 1.56 ($P < 0.01$) times, respectively, relative to the values of rats with AH. Activation of both the mitochondrial and immune pathways of apoptosis was found. The above parameters significantly exceeded the values of intact rats by 1.59 ($P < 0.01$), 1.63 ($P < 0.01$) and 1.68 ($P < 0.01$) times, respectively. Along with this, an even greater inhibition of cytochrome P450, e-NOS activity, and a sharp increase in the activity of iNOS and its metabolic products were noted.

Thus, the conducted studies have shown that when the nitroergic system inducer L-arginine is administered to animals with AH, a decrease in the NO level is characterized by iNOS inhibition and an increase in e-NOS activity. A decrease in iNOS activity is associated with increased microcirculation in tissues, oxygen supply to tissues, reduction of hypoxia, main mechanism of

formation of free radicals, reactive oxygen species, and apoptosis of hepatocytes.

When a non-selective L-NAME inhibitor is administered to animals, NO overexpression is provided by increased iNOS activity. The direction of activity of liver MOX enzymes is determined by the intensity of eNOS and iNOS, as well as by the expression of ONO22-. Hyperproduction of NO can have a damaging effect on the cell due to direct and indirect mechanisms. The most important mechanism of the mediated damaging effect of excess NO is considered to be the interaction of NO with the superoxide anion to form peroxynitrite. Peroxynitrite modifies and breaks DNA strands, while simultaneously inhibiting DNA ligase, which causes even more damage to the DNA and the cell as a whole.

Assessing the relationship between the NO system and cytochrome P450, it should be emphasized that the balanced functioning of these systems plays an important role in the regulation of programmed cell death, expression of genes involved in cell cycle blockade, Bcl-2 modulation, and DNA fragmentation. An important place is given to the P53 protein, which protects the genome. Normally, the concentration of P53 in the cell is very low and it quickly degrades. DNA damage leads to the accumulation of P53. Accumulation of P53 during cell death caused by NO was detected. It turned out that L-arginine lowers and L-NAME increases the level of P53, which indicates an active role of NO in this process.

The pro-apoptotic effect of NO on liver tissue is realized directly through the mechanisms of caspase activation. One of the ways to activate caspases is to release cytochrome c and other factors from the mitochondria into the cytoplasm. L-arginine lowers the level of NO by inhibiting iNOS, reduces the release of cytochrome a into the blood, in animals with AH, L-NAME-on the contrary, increases the level of this cytochrome in the blood to an even greater extent. Tumor necrosis factor TNF- α induced NO synthesis, followed by a decrease in cytochrome P450 levels. The inhibition of this hemoprotein by TNF α is directly related to the induction of iNOS. L-arginine reduced high TNF α levels in AH rats. The L-NAME inhibitor increased its level even more.

4. Conclusion

Animals with AH showed an increase in the level of P53 and TNF α , the release of cytochrome C from the mitochondria. Injection of the drug metabolism inducer

benzonal at a dose of 50 mg / kg to animals with AH (CC14) significantly reduced the phenomena of apoptosis, and the use of cimetidine at a dose of 10 mg/kg potentiated apoptosis of hepatocytes, dramatically increasing the content of P53, TNF α , and cytochrome C in biological fluids. The NO inducer L-arginine reduced the level of pro-apoptotic parameters in animals with AH. Nonselective NOS inhibitor L-NAME increased the level of P53 protein, TNF α , and cytochrome C output from hepatocyte mitochondria compared to the AH group.

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